Modulation of Dopamine D$_2$ Receptor Signaling by Actin-Binding Protein (ABP-280)

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ABSTRACT

Proteins that bind to G protein-coupled receptors have recently been identified as regulators of receptor anchoring and signaling. In this study, actin-binding protein 280 (ABP-280), a widely expressed cytoskeleton-associated protein that plays an important role in regulating cell morphology and motility, was found to associate with the third cytoplasmic loop of dopamine D$_2$ receptors. The specificity of this interaction was originally identified in a yeast two-hybrid screen and confirmed by protein binding. The functional significance of the D$_2$ receptor-ABP-280 association was evaluated in human melanoma cells lacking ABP-280. D$_2$ receptor agonists were less potent in inhibiting forskolin-stimulated cAMP production in these cells. Maximal inhibitory responses of D$_2$ receptor activation were also reduced. Further yeast two-hybrid experiments showed that ABP-280 association is critically dependent on the carboxyl domain of the D$_2$ receptor third cytoplasmic loop, where there is a potential serine phosphorylation site (S358). Serine 358 was replaced with aspartic acid to mimic the effects of receptor phosphorylation. This mutant (D$_2$S358D) displayed compromised binding to ABP-280 and coupling to adenylyl cyclase. PKC activation also generated D$_2$ receptor signaling attenuation, but only in ABP-containing cells, suggesting a PKC regulatory role in D$_2$-ABP association. A mechanism for these results may be derived from a role of ABP-280 in the clustering of D$_2$ receptors, as determined by immunocytochemical analysis in ABP-deficient and replete cells. Our results suggest a new molecular mechanism of modulating D$_2$ receptor signaling by cytoskeletal protein interaction.

Dopamine D$_2$ receptors, which belong to the superfamily of G protein-coupled receptors (GPCRs), regulate essential neurobiological and endocrine processes. Hence, the cellular signaling of D$_2$ receptors has been extensively investigated. Beyond inhibiting adenylate cyclase, D$_2$ receptors modulate the activities of potassium and calcium channels, phospholipase C, mitogen-activated protein kinase, sodium-proton exchangers, and the release of arachidonic acid (Sibley and Monsma, 1992; Civelli et al., 1993; Neve and Neve, 1997; Missale et al., 1998). These D$_2$ signaling pathways seem to be regulated in a complex fashion. For instance, protein kinase C (PKC) phosphorylation directs the preferential coupling of D$_2$ receptors from the inhibition of adenylate cyclase to the release of arachidonic acid (Di Marzo et al., 1993). Cell type specificity is exhibited as D$_2$ receptors inhibit phospholipase C in pituitary cells, but activate it in fibroblast cells (Vallar et al., 1990). The effector coupling efficiency of D$_2$ receptors seems also to be regulated by the subcellular localization of these receptors. In particular, presynaptic D$_2$ autoreceptors found on the axonal terminals of dopaminergic neurons are more sensitive to agonist stimulation than their identical postsynaptic counterparts (Skirboll et al., 1979; Clark and White, 1987; Missale et al., 1998). In light of these differences in both the cell-type specificity and coupling efficiency of D$_2$ receptors, cellular pathways additional to G protein coupling are likely to be involved in the activity and regulation of D$_2$ receptor signaling.

Effecter coupling and membrane targeting of GPCRs have been found to be regulated by a variety of protein-protein interactions. A nearly universal mechanism of terminating GPCR signaling is mediated by the binding of arrestins after receptor phosphorylation by GPCR kinases (Krupnick and Benovic, 1998). Recently, proteins that bind to specific members of GPCRs have been identified as unique players in receptor signaling or targeting. For example, the association of β$_2$ adrenergic receptors with the protein translation initiation factor (eIF-2B) has been shown to enhance the ability of these receptors to activate adenylate cyclase (Klein et al., 1997). β$_2$ adrenergic receptors also activate sodium-proton exchangers by recruiting regulatory factors in an agonist-dependent but G protein-independent fashion, indicating the

ABBREVIATIONS: GPCR, G protein coupled receptor; PKC, protein kinase C; ABP-280, actin-binding protein 280; MBP, maltose-binding fusion protein; GST, glutathione S-transferase; CHO, Chinese hamster ovary; TBS-T, Tris-buffered saline/Tween 20; TEM, Tris/EDTA/MgCl$_2$; 6,7-ADTN, (±)-2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene hydrobromide; PMA, 4β-phorbol-12-myristate-13-acetate; FITC, fluorescein isothiocyanate; PDZ, PSD-95/DlgA/ZO-1.
existence of novel signaling mechanisms distinct from traditional GPCR second messenger pathways (Hall et al., 1998). Moreover, a family of single-transmembrane-domain proteins has been identified as modifying proteins for calcitonin-receptor-like receptors (McLatchie et al., 1998). These single-transmembrane-domain proteins are required for the targeting of calcitonin-receptor-like receptors to the plasma membrane and also determine their ligand specificity. Finally, ATRAP, a novel protein that interacts with the carboxyl-terminal cytoplasmic domain of the angiotensin II type 1 receptor has been found to negatively regulate receptor signaling (Daviet et al., 1999).

We have investigated the possibility that novel protein interactions with the D₂ receptor may regulate its signaling or targeting. Here, we show that cytoskeletal protein actin-binding protein 280 (ABP-280) interacts with the third cytoplasmic loop of the D₂ receptor. We demonstrate that this association enhances coupling efficiency of D₂ receptors to adenylate cyclase, can be regulated by PKC activation, and acetate method. The final transformation mixture (2.6 plasmid of the D₂ receptor. We demonstrate that this association enhances coupling efficiency of D₂ receptors to adenylate cyclase, can be regulated by PKC activation, and acetate method. The final transformation mixture (2.6

Materials and Methods

Yeast Two-Hybrid. The third cytoplasmic loop of the human D₂ receptor (residues 211–372) was amplified by PCR and subcloned in-frame into the Gal4 DNA-binding domain vector pGBT9 (Clontech, Palo Alto, CA) to generate pGBT9-D₂. pGBT9-D₂ was used to screen a human brain cDNA library constructed in the Gal4 activation domain vector pACT2 (Clontech). Library plasmid DNAs were isolated by plating 5 × 10⁶ independent clones on 80 150-mm LBA plates. Handling and transformation of yeast (strain Y190) were performed as described (Matchmaker Two-hybrid System protocol; Clontech). Briefly, yeast cells were sequentially transformed with pGBT9-D₂ and 300 μg of library plasmid DNA using the lithium acetate method. The final transformation mixture (2.6 × 10⁶ yeast plasmid transformants) was plated onto 100 150-mm LBA plates. Robust colonies were restreaked on fresh plates and tested for in-frame into the Gal4 DNA-binding domain vector pGBT9 (Clontech). Library plasmid DNAs were isolated by plating 5 × 10⁶ independent clones on 80 150-mm LBA plates. Handling and transformation of yeast (strain Y190) were performed as described (Matchmaker Two-hybrid System protocol; Clontech). Briefly, yeast cells were sequentially transformed with pGBT9-D₂ and 300 μg of library plasmid DNA using the lithium acetate method. The final transformation mixture (2.6 × 10⁶ yeast plasmid transformants) was plated onto 100 150-mm LBA plates. Robust colonies were restreaked on fresh plates and tested for

In Vitro Protein-Protein Binding. The third cytoplasmic loops of D₁, D₄, D₉, and D₁₄ receptors were subcloned in frame into bacterial expression vector pGEX-3X (Pharmacia, Piscataway, NJ) to generate glutathione S-transferase (GST) fusion proteins (note: use of the D₄ receptor in all experiments was limited to the D₄ isoform). A single bacterial colony was used to inoculate 2 × YTA medium and incubated at 30°C until an A₆₀₀ of 0.3 was reached. Isopropyl-β-thiogalactopyranoside (100 μM) was then added to induce the formation of fusion proteins. Bacteria were pelleted at 5,000 g for 5 min, resuspended in PBS, and lysed by sonication. After centrifugation两次 at 13,000 g for 15 min at 4°C, supernatant was collected as the bacterial lysate. ABP-280 (residues 1779–2143) was purified as a maltose-binding fusion protein (MBP) with amylose affinity chromatography (Sigma, St. Louis, MO) as a substrate.

Receptor Radioligand Binding. Stably transfected cells were grown to confluence on 150-mm tissue culture plates. Cells were rinsed with 5 ml of ice-cold PBS once and scraped off the plates in the TEM buffer (25 mM Tris·HCl, pH 7.4, 1 mM EDTA, and 6 mM MgCl₂). After centrifugation at 1200 g for 3 min at 4°C, cell pellets were frozen at −70°C until the day of assay. Cell pellets were resuspended in ice-cold TEM buffer at a concentration of 200 to 400 μg of total protein/ml and homogenized using a polytron homogenizer (Brinkmann Instruments, Westburg, NY) at a setting of 5 for 10 sec on ice. Radioligand binding was performed in a volume of 1 ml using approximately 200 μg of total protein per tube. Competition binding assays were performed using 0.1 to 0.3 nM [³H]spiperone (99 Ci/mmol; Amersham) and various concentrations of competing compounds. Reaction mixtures were then incubated for 1 h at room temperature and terminated by rapid vacuum filtration through GF/B filters presoaked in 0.5% polyethylenimine (Sigma) using a 24-port harvester (Brandel, Montreal, Canada). Filters were immediately washed with 5 ml of TEM buffer, air dried, and individual filter discs were placed in counting vials with 5 ml of scintillation fluid for counting in a Beckman LS-6800 liquid scintillation counter. Data were analyzed using GraphPad software (San Diego, CA).
was carried out at 37°C for 30 min and terminated by the addition of ice-cold 70% ethanol. cAMP samples were collected in Eppendorf tubes. After drying down, cAMP levels in the samples were determined using a sensitive succinylation method. Each cAMP sample was dissolved in 1 ml of ice-cold NaOAc (50 mM, pH 6.2). One hundred microliters of the sample were succinylated by incubating with 15 mM succinic anhydride (dissolved in 25% triethylamine/75% acetone) on ice for 10 min. Succinylation was terminated by the addition of 2 ml of ice-cold NaOAc (50 mM). One hundred microliters of the dilute succinylated sample were incubated with 100 μl anti-cAMP antibody (Sigma) for 4 h at 4°C. An aliquot of 125I-cAMP (0.0045 μCi) (NEN, Boston, MA) was added into each sample. After overnight incubation at 4°C, immunocomplexes were precipitated with 100 μl of 10% bovine serum albumin and 100 μl of 95% ice-cold ethanol. Radioactivity was determined by counting in a Beckman 5500 Gamma counter. This succinylation radiolmmunoassay has a detection limit of 100 fmol cAMP. Data were analyzed using GraphPad software.

**Immunocytochemistry.** A7 and M2 melanoma cells grown in 35-mm glass-bottomed culture dishes (MatTek, Ashland, MA) were transiently transfected with amino-terminally FLAG-tagged D2 or D1 receptors. Two days after transfection, cells were washed in PBS and subsequently fixed in 3.7% formaldehyde in PBS for 30 min on ice. After three washes with PBS, cells were blocked in 5% BSA for

### TABLE 1

Protein-protein interactions detected with the yeast two-hybrid assay

<table>
<thead>
<tr>
<th>HHS3</th>
<th>β-Gal</th>
<th>ONPG</th>
</tr>
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<tbody>
<tr>
<td>pACT2-ABP:pGBT9-D2 (211–372)</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>pGBT9-ABP:pACT2-D2 (211–372)</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>pACT2-ABP:pGBT9-D2 (211–343)</td>
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<td>++</td>
</tr>
<tr>
<td>pACT2-ABP:pGBT9-D2 (211–388)</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>pACT2-ABP:pGBT9-D3 (210–375)</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>pACT2-ABP:pGBT9-D4 (214–346)</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>pACT2-ABP:pGBT9-D7</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>pACT2-ABP:pGBT9-D3s58D</td>
<td>+</td>
<td>+</td>
</tr>
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</table>

ONPG: o-nitrophenyl β-D-galactopyranoside.

* P < .05, D2S58D versus D2, unpaired Student’s t test.

**Fig. 1.** Binding of ABP-280 to GST-D2 in vitro. Bacterial lysates containing GST-D1, GST-D2, GST-D3, and GST-D4 fusion proteins were tested for their abilities to bind to purified MBF-ABP280. Lanes 1 to 4 indicate GST fusion proteins from bacterial lysates (Lysate). Lanes 5 to 8 indicate GST fusion proteins retained after incubation with MBP-ABP280 (Bound). GST-D2 and GST-D3, but not GST-D1 or GST-D4, readily bound to MBP-ABP280.

### TABLE 2

Ligand affinity of dopamine D2 receptor in M2, A7, and CHO cells

<table>
<thead>
<tr>
<th>M2</th>
<th>A7</th>
<th>CHO</th>
</tr>
</thead>
<tbody>
<tr>
<td>K&lt;sub&gt;i&lt;/sub&gt; (μM)</td>
<td>25.175</td>
<td>28.455</td>
</tr>
<tr>
<td>Dopamine</td>
<td>8,900 ± 2,630</td>
<td>10,100 ± 4,970</td>
</tr>
<tr>
<td>Dopamine + GTP&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.175</td>
<td>28.455</td>
</tr>
<tr>
<td>6,7ADTN</td>
<td>350 ± 70</td>
<td>330 ± 120</td>
</tr>
<tr>
<td>(−)-Sulpiride</td>
<td>170 ± 40</td>
<td>160 ± 10</td>
</tr>
<tr>
<td>Haloperidol</td>
<td>3.16 ± 0.29</td>
<td>2.41 ± 0.47</td>
</tr>
</tbody>
</table>

<sup>a</sup>GTP concentration was 50 μM; K<sub>i</sub> was calculated as the average of two experiments.

ND, not determined.

**Fig. 2.** D2 receptor-mediated inhibition of cAMP accumulation in M2, A7, and CHO cells. Dose-response curves for (A) dopamine or (B) 6,7-ADTN inhibition of cAMP accumulation were determined on cells stimulated with 10 μM forskolin and increasing concentrations of dopamine or 6,7-ADTN. Results are expressed as percentage inhibition of forskolin-stimulated cAMP level. Curves are plotted as average ± S.E. from three to five independent experiments. D2 receptor expression levels were 2.48 pmol/mg of protein (M2) versus 2.55 pmol/mg of protein (A7). The stable CHO cell clone used contained receptor levels of 1.07 pmol/mg of protein. No significant change in either agonist or antagonist affinity for D2 receptors was observed in M2, A7, or CHO cells.

A7-D2 | M2-D2 | CHO-D2

**A.**

**B.**
1 h and treated with M2 anti-FLAG antibody (10 μg/ml; Eastman Kodak, New Haven, CT) in 5% BSA overnight at 4°C. The plates were then washed three times with PBS and treated with fluorescein isothiocyanate (FITC)-conjugated goat antimouse IgG (Santa Cruz Biotech) for 1 h. The antibody-labeled cells were then rinsed three times for 5 min each before viewing by confocal microscopy. Confocal microscopy was performed on a Bio-Rad MRC confocal laser microscope equipped with a Nikon Diaphot 200 inverted microscope using a Nikon 60 × 1.40 NA oil-immersion objective. FITC was excited with a 488-nm argon/krypton laser and emitted fluorescence was detected with a 515–540 nm band pass filter.

Statistical Analysis. Statistical analysis was carried out by unpaired Student’s t test.

### Results

**Binding of ABP-280 to the Third Cytoplasmic Loop of the Dopamine D2 Receptor.** To identify proteins that bind to the dopamine D2 receptor, a yeast two-hybrid screen was performed using the entire third cytoplasmic loop of the human D2 receptor (long form, residues 211–372). A single clone (represented identically in three distinct colonies) encoding part of ABP-280 (residues 1779–2134, known as ABP repeats 16 to 19) was isolated from a human brain cDNA library. The specificity of the D2-ABP-280 interaction was evaluated by examining the ability of ABP-280 to bind to the entire third cytoplasmic loops of other dopamine receptors. The short form of the D2 receptor bound to ABP-280 to a similar degree as that of the long form (Table 1). Interestingly, the third cytoplasmic loop of the dopamine D3 receptor also interacted strongly with ABP-280. As quantified by the activities of β-galactosidase, ABP-280 bound to the D3 receptor more intensely than the D2 receptor (Table 1). ABP-280 was found not to interact with the third cytoplasmic loops of either the D4 receptor, a third member of the D2 receptor subfamily, or the D1 receptor, which couples to the stimulation rather than inhibition of adenylate cyclase (Table 1).

The specific interaction of D2 and D3 receptors with ABP-280 was further verified by in vitro protein-protein binding. An MBP-ABP280 (residues 1779–2134) fusion protein was purified and incubated with crude GST fusion proteins containing the third cytoplasmic loops of D2, D3, D4, and D1 receptors. Figure 1 shows that GST-D3 and GST-D4 readily bound to MBP-ABP280, whereas no interaction was observed with GST-D1 and GST-D2 fusion proteins.

**The Association of D2 Receptors with ABP-280 is Essential for the Efficient Coupling of the Receptor to the Inhibition of Adenylate Cyclase.** The availability of human melanoma cell line (M2) that does not express ABP-280 endogenously allows us to assess D2 receptor properties in the absence of ABP-280. As a control, the A7 cell line was generated by stably transfecting M2 cells with ABP-280 cDNA. Clones stably expressing similar levels of D2 receptors in M2 and A7 cells were selected for further receptor binding and activation studies. Radioligand binding experiments revealed similar affinity for several agonists and antagonists in M2, A7, and control D2 transfected CHO cells (Table 2). Fifty-micromolar GTP treatment reduced the dopamine affinity similarly on both M2 and A7 cells, indicating that ABP-280 binding did not seem to affect receptor/G protein interaction directly (Table 2). However, a significant difference was observed in agonist-mediated inhibition of forskolin-stimulated cAMP production. Figure 2 shows that dopamine was less potent in inhibiting forskolin-stimulated adenylate cyclase in M2 than in A7 cells (EC_{50} = 33.8 and 3.1 nM, respectively; Table 3). The maximum inhibition of D2 receptor activation by dopamine was also reduced in M2 cells (42% compared with 78% in A7 cells; Fig. 2, Table 3). Similar results were observed with a synthetic D2 agonist 6,7-ADTN (Fig. 2, Table 3). As a control study, we found that both the potency and maximal inhibition of dopamine in A7 cells were comparable with those found using a CHO cell line (Fig. 2, Table 3) and to published results using other cell lines (Missale et al., 1998). These findings indicate that the D2 receptor is less efficient in coupling to the inhibition of adenylate cyclase in the absence of ABP-280.

**Regulation of the D2 Receptor and ABP-280 Interaction by Protein phosphorylation.** GPIbα of the GP Ib-IX complex, the platelet von Willebrand factor receptor that mediates the initial attachment of platelets at a site of injury, has been shown to bind to the same region of ABP-280 as does the D2 receptor (Meyer et al., 1998). GPIbα requires a 30-amino acid domain at its carboxyl terminus for ABP association (Andrews and Fox, 1992). This region displays considerable homology with the carboxyl domain of the D2 and D3, but not the D1 or D4, third cytoplasmic loops (Fig. 3). We tested whether this receptor region is critical for binding to ABP-280. Table 1 shows that elimination of this stretch (D2A314–368) abolished the ability of the D2 receptor to associate with ABP-280. Within this domain, there exists a conserved serine residue that is a putative PKC phosphorylation site. Table 1 shows that the D2S358D mutant receptor in A7 and D2S358D mutants of CHO cells does not express ABP-280. Therefore, we replaced this serine residue with aspartic acid (D2S358D) to mimic its phosphorylated state. Table 1 shows that the D2S358D mutant receptor

### Table 3

<table>
<thead>
<tr>
<th>D2 receptors</th>
<th>EC_{50} (nM)</th>
<th>Maximum Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dopamine</td>
<td>6,7-ADTN</td>
<td>Dopamine</td>
</tr>
<tr>
<td>A7-D2</td>
<td>3.09 ± 0.46 (3)</td>
<td>0.28 ± 0.06 (5)</td>
</tr>
<tr>
<td>M2-D2</td>
<td>33.78 ± 1.08 (4)*</td>
<td>4.90 ± 0.73 (3)*</td>
</tr>
<tr>
<td>CHO-D2</td>
<td>3.17 ± 0.50 (3)</td>
<td>ND</td>
</tr>
</tbody>
</table>

*P < .05, compared with A7-D2 or CHO-D2 cells, unpaired Student’s t test.

ND, not determined.
displayed significantly reduced binding to ABP-280 compared with the wild-type receptor.

To elucidate further the functional significance of serine-358, we stably transfected D₂S₃₅₈D receptors into A₇ and M₂ cells. Figure 4A shows that the agonist potency of D₂S₃₅₈D was impaired in A₇ cells, with a reduction also in the maximal inhibition of forskolin-stimulated cAMP production (Fig. 4A, Table 4). In M₂ cells, however, no shifts in agonist potency or maximal signaling between wild-type and D₂S₃₅₈D receptors were observed, indicating that the effects of this mutation are specific only to ABP-containing cells (Fig. 4B; Table 4). Thus, a structural mimic of serine-358 phosphorylation negatively regulates D₂ receptor association with ABP-280 as well as D₂ receptor signaling in a parallel fashion. Next, we examined the effects of PKC activation on D₂ signaling. Table 4 shows that PMA treatment reduced the agonist potency of D₂ in A₇ but not in M₂ cells. This reduction in potency was similar to that of the D₂S₃₅₈D phosphorylation mimic observed in A₇ cells. Notably, there was no further shift in agonist potency of the D₂S₃₅₈D receptor on treatment with PMA (Table 4). Hence, PKC activation can modulate D₂ receptor signaling in a manner consistent with a regulatory role in ABP-280-D₂ association.

**ABP-280 Affects the Cell Surface Expression Pattern of the D₂ Receptor.** Several intracellular proteins known to

<table>
<thead>
<tr>
<th></th>
<th>EC₅₀ (nM)</th>
<th>Maximum Inhibition (%)</th>
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<tbody>
<tr>
<td>A₇-D₂</td>
<td>0.25 ± 0.10 (4)</td>
<td>79.50 ± 4.29 (4)</td>
</tr>
<tr>
<td>A₇-D₂ + PMA</td>
<td>0.83 ± 0.13 (3)*</td>
<td>81.18 ± 0.96 (3)</td>
</tr>
<tr>
<td>A₇-D₂S₃₅₈D</td>
<td>0.75 ± 0.15 (4)*</td>
<td>64.84 ± 2.73 (4)*</td>
</tr>
<tr>
<td>A₇-D₂S₃₅₈D + PMA</td>
<td>1.24 ± 0.37 (3)</td>
<td>72.60 ± 2.44 (3)</td>
</tr>
<tr>
<td>M₂-D₂</td>
<td>3.95 ± 0.67 (4)</td>
<td>34.30 ± 4.71 (4)</td>
</tr>
<tr>
<td>M₂-D₂ + PMA</td>
<td>4.03 ± 0.98 (3)</td>
<td>40.32 ± 5.20 (3)</td>
</tr>
<tr>
<td>M₂-D₂S₃₅₈D</td>
<td>6.13 ± 0.95 (3)</td>
<td>39.40 ± 3.57 (3)</td>
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</tbody>
</table>

*P < 0.05, compared with A₇-D₂ cells, unpaired Student’s t test.

**Fig. 3.** Alignment of the peptide sequences from the region of GPIbα that is known to bind to ABP-280, and the C-terminal region of the third cytoplasmic loop of D₁ to D₄ receptors. All the peptides were compared with GPIbα peptide. Gray boxes depict conserved amino acid residues. A putative PKC phosphorylation site in D₂ and D₃ receptors was indicated in bold.

**Fig. 4.** Dose-response curves of 6,7-ADTN-stimulated inhibition of cAMP accumulation in wild-type (D₂) and mutant (D₂S₃₅₈D) D₂ receptors. Both receptors were stably transfected into A₇ (A) and M₂ (B) cells. Stable clones with similar receptor expression levels were tested. The expression levels were 1.48 pmol/mg of protein (A₇-D₂) versus 1.6 pmol/mg of protein (A₇-D₂S₃₅₈D) and 1.45 pmol/mg of protein (M₂-D₂) versus 1.3 pmol/mg protein (M₂-D₂S₃₅₈D). Dose-response curves were determined as in Fig. 2. Data shown are the mean ± S.E. from three independent experiments.

**Fig. 5.** Plasma membrane expression of FLAG-D₁ and FLAG-D₂ receptors in transiently transfected A₇ and M₂ cells. D₂ receptor expression levels on A₇ and M₂ cells in this specific experiment are 1.2 pmol/mg protein and 1.0 pmol/mg protein, respectively. FLAG-D₂ receptors displayed membrane clustering in A₇ cells (A), but more uniform surface distribution in M₂ cells (B). In contrast, FLAG-D₁ receptors showed a similar clustering appearance in both A₇ (C) and M₂ (D) cells. Representative viewing fields were chosen from three independent experiments.
interact with cell surface receptors have been found to be involved in receptor targeting (Gomperts, 1996). To visualize the cellular distribution of D2 receptors, we attached a FLAG epitope to the amino-terminus of the receptors (Vickery and von Zastrow, 1999). FLAG-tagged D2 receptors expressed on A7 and M2 cells showed similar affinity to agonist 6,7-ADTN compared with untagged D2 receptors ($K_i = 284 \pm 72$ and $222 \pm 46$ nM, respectively; $n = 3$; Table 2). FLAG-tagged D2 were then transiently transfected into A7 and M2 cells, labeled with FITC-conjugated secondary antibodies and visualized by confocal microscopy. D1 receptors, also tagged with FLAG at the amino terminus, were used as control receptors. Both D2 and D3 receptors displayed clustering in A7 cells (Fig. 5, A and C). However, in ABP-280-deficient M2 cells, although D1 receptors maintained a clustering appearance, D2 receptors were more uniformly distributed along the plasma membrane (Fig. 5, B and D). These results indicate that ABP-280 contributes to D2 receptor clustering on the cell surface and may serve to anchor these receptors in prime locations for efficient cellular response to agonist stimulation.

**Discussion**

In this study, we found that the dopamine D2 receptor binds to ABP-280, a cytoskeleton-associated protein. Several other neuroreceptors have been reported to associate with cytoskeletal elements. For instance, subtypes of the N-methyl-D-aspartate receptor have been shown to bind to $\alpha$-actinin-2, also a member of the actin-binding protein family, in postsynaptic densities of cortical neurons (Wyszynski et al., 1997). In addition, several ligand-gated ion channels are known to be linked to the cytoskeleton via different adaptor proteins (Kirsch and Betz, 1993; Carbonetto and Lindenbaum, 1995; Wang et al., 1999). Most interestingly, recent reports suggest a similar linkage between GPCRs and the cytoskeleton. For example, the carboxyl terminus of the somatostatin receptor has been shown to associate with the cytoskeleton via cortactin-binding protein (Zitzer et al., 1999). Our data indicate that D2 receptors can bind directly to ABP-280 with important functional consequences.

The association between the D2 receptor and ABP-280 was shown to enhance receptor signaling. In M2 melanoma cells that do not express ABP-280, the ability of D2 receptors to inhibit forskolin-stimulated adenylate cyclase activity was greatly reduced. We have further shown that mimicking protein phosphorylation by substitution of serine-358 with aspartic acid within the ABP-association domain reduces the binding ability and signal coupling efficiency of the mutant D2 receptor (Table 1, Table 4). Direct stimulation of PKC with PMA also reduces D2 signaling (Table 4). This phosphorylation regulation thus suggests a dynamic feature of receptor-cytoskeletal interactions and their potential for regulation by physiological stimuli. PKC activation can occur through multiple pathways. In particular, many GPCRs are capable of signaling homologous and heterologous receptor desensitization through PKC-dependent mechanisms (Chuang et al., 1996). However, it is not clear exactly how PKC-mediated phosphorylation mechanisms alter receptor-signaling pathways. Our findings suggest that one of these mechanisms may involve a regulated association with cytoskeletal components.

It is well known that D2 autoreceptors are more sensitive to agonists than their postsynaptic counterparts (Skirboll et al., 1979), but the underlying mechanisms for this differential sensitivity remain elusive. The involvement of distinct D2 receptor protein sequences is unlikely (Skirboll et al., 1979; Clark and White, 1987; Missale et al., 1998). Our results indicate that the differential agonist sensitivity of the presynaptic and postsynaptic D2 receptors may result from differential neuronal compartmental interactions between D2 receptors and ABP-280. It would be interesting to examine whether the in vivo interaction between ABP-280 and the D2 receptor is more prevalent presynaptically than postsynaptically.

The exact mechanism by which ABP-280 modulates the signaling of D2 receptors is still under investigation, although it is likely that this cytoskeletal component acts as a scaffolding protein. By clustering the components of the signaling pathways together, scaffolding molecules can greatly increase the efficiency of receptor-effector coupling. It has been shown that in *Drosophila melanogaster*, INAD, a protein with five distinct PSD-95/DlgAZO-1 (PDZ) domains, serves as a scaffold in the assembly of a highly organized phototransduction pathway that includes receptor, effectors, and regulators, thereby endowing the signaling pathway with extremely high fidelity (Montell, 1998). Our data indicate that ABP-280 assists in the clustering of D2 receptors to specific cell surface locales. Whether these regions are also enriched in signaling intermediates of D2 receptors remains to be determined. Studies have demonstrated that the distribution of G proteins in the plasma membrane is not random (Wang et al., 1989) and that G proteins or adenylyl cyclase could also be attached to components of the cytoskeleton (Graeser and Neubig 1993; Neubig, 1994). Thus by anchoring receptors as well as signaling molecules, the cytoskeleton may ensure rapid and efficient signal transduction.

ABP-280 is an abundant cytoplasmic protein with an amino terminal actin-binding domain of approximately 275 amino acids, followed by 24 tandem repeats, each approximately 96 amino acids in length (Gorlin et al., 1990). In addition to organizing actin fibers, distinct repeats of ABP-280 have been shown to interact with a number of membrane proteins, including GPlbα, the $\alpha$ subunit of glycoprotein Ib (Andrews and Fox, 1992; Meyer et al., 1998), integrin (Sharma et al., 1995), furin (Liu et al., 1997), as well as the cytosolic stress-activated protein kinase kinase (Marti et al., 1997). Interestingly, these interactions occur in distinct ABP repeat domains with unique functional consequences, ranging from effects on cytoskeletal organization to the inhibition of receptor internalization and effector coupling. Notably, the carboxyl-terminal repeat (number 24) of ABP-280 contains a self-assembly sequence that forms homodimers. Conceivably, these structural features and multiple interaction domains of ABP-280 may enable the formation of receptor-effector complexes necessary for the efficient signaling of D2 receptors and their proper membrane targeting, as our results suggest.

Recently, the third cytoplasmic loop of D2 receptors was shown to interact with another protein, spinophilin (Smith et al., 1999). Although the functional significance of this association is still unknown, it has been suggested that, like ABP-280, spinophilin may play a role as a scaffolding protein in organizing the D2 receptor-signaling complex. Spinophilin is also a cytoskeletal-associated protein; it contains an actin-
binding domain (Satoh et al., 1998). In addition, spinophilin has a carboxyl-terminal coiled-coil structure and a single consensus PDZ domain. It has been shown that spinophilin interacts with protein phosphatase-1 at a site distinct from the D₂ binding site. Hence, the multiplicity of G protein-independent interactions with the D₂ receptor seem to enable a diversity of functional regulatory processes, ranging from the inhibition of adenylate cyclase, as we have shown, to the potential phosphatase-mediated activity against competing stimulatory kinase pathways.

In conclusion, we have identified a novel association between the D₂ receptor third cytoplasmic loop and the actin cytoskeleton via ABP-280. This association enhances receptor signaling and can be regulated by protein phosphorylation. Further studies to establish this association in neuronal cells will be of great value in understanding the role of the cytoskeleton in dopaminergic neurotransmission.

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